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46. R.A.P.I.D. - PCR (LightCycler) IN DIAGNOSIS OF BIOLOGICAL AGENTS

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ABSTRACT

Although the historic use of biological weapons has been infrequent, a belief that state sponsored armies or terrorist organizations will use this type of weapon has never been greater which demands a capability for rapid medical response and early intervention. Molecular diagnostic methods, based on DNA amplification known as PCR (Polymerase Chain Reaction) are promising tools in fast and specific detection and identification of biological agent(s).

The R.A.P.I.D.TM - PCR (Ruggedized Advanced Pathogen Identification Device) (fig.1), is a 32 sample capacity, automated instrument integrating Idaho Technology's LightCycler® technology into a portable, impact resistant package. This allows field identification of pathogens quickly. Monitoring the fluorescence from the double-stranded DNA dye SYBR® Green (fig.2), followed by differentiation of products by melting curves or from TaqMan® probes (6-FAM-oligo-TAMRA), allows inexpensive quantification of low initial template copy number. Cycle sequencing reactions done in the thermocycling module of the R.A.P.I.D. system are faster, cleaner and more readable than parallel reactions done in a conventional heat block cycler. The use of air as the cycling medium ensures temperature uniformity and rapid heat exchange with the sample loaded in thin micro-capillary tubes, which are ideally suited to temperature cycling, because of their extremely high surface area to volume ratio. A conventional PCR protocol takes up-to 3 hours to do 30 three-temperature cycles. The R.A.P.I.D. system can complete a 40-cycle reaction in less than 20 minutes (6 to 30 min.). This makes R.A.P.I.D. system the fastest thermal cycler in the world. The United States Air Force has developed over 50 assays for infectious agents on the R.A.P.I.D. system. Assays for infectious agents typically consist of two temperatures cycling for 40 cycles. Protocols for isolation of bacteria and viral DNA (or RNA) have been developed for clinical specimens, air samples and water samples. Protocols for food samples are being developed now. Assays in use for: *Bacillus anthracis*, *Yersinia pestis*, *Clostridium botulinum*, *Staphylococcus aureus*, *Francisella tularensis*, *Salmonella*, *Shigella*, *Vibrio cholerae*, *E. coli*, *Campylobacter*, *VEE*, *West Nile*, *Yellow Fever*, *Brucella spp.*, and many others. The continuing effort and advances in testing design make this capability an asset for Army Commanders and Medical facilities alike.

INTRODUCTION

Of all weapons of mass destruction, biological weapons (BW) today present the greatest danger (1). Although the historic use of BW has been infrequent, a belief that state sponsored armies or terrorist organizations, groups or individuals, will use this type of weapon has never been greater which demands a capability for rapid medical response and early intervention (2). Approximately 17 countries, (including five implicated as sponsors of international terrorism), may have active research and development programs for BW (3). Protecting the armed forces against BW, is less difficult than protecting the civilian population. The armed forces are relatively small populations that can be vaccinated against the major threat agents. Aerosols containing biologic materials can be detected at a distance,

and protective masks and suits are effective. Military medical personnel are trained to recognize and treat casualties. The civilian population cannot be protected in the same manner as the armed forces. Terrorist organization may not be deterred, may evade detection and may succeed in releasing an aerosol of a virulent biological agents (BA) in a susceptible target area (4). The number of false alarms and hoaxes involving BA keeps increasing and cost societies a lot. The economic impact of bio-terrorist attack can range from \$477.7 million per 100.000 persons (exposed to brucellosis scenario) to \$26,2 billion per 100.000 persons (exposed to anthrax scenario) (5). Unusual outbreaks of illness might be essential suspicion in recognizing of deliberate releasing of a biological agents (6). The specter of potential BA is well known and includes: anti-human, anti-plant and anti-animal agents(7,8). There are many potential anti-human BA: I. Bacterial agents: *Bacillus anthracis*, *Brucella spp.*, *Vibrio cholerae*, *Yersinia pestis*, *Francisella tularensis*, *Salmonella spp.*, *Shigella spp.*, II. Viruses: *Variola major*, Filoviruses and Arenaviruses, III. Rickettsiae: *Coxiella burnetii*, III. Biological toxins: Botulinum, Staphylococcal enterotoxin-B, Mycotoxins. Certain agents incapacitate, whereas others kill (up to 90% mortality rate) (3). Routes of infection could be: respiratory (aerosols as most effective delivery method), skin and gastrointestinal (food, water).

Rapid response to BW attack depends on rapid identification of BA that includes different methods such as: immunofluorescence assay, enzyme-linked immunoassay, immunochromatographic assay and Nucleic acid detection.

METHOD AND DISCUSSION

Molecular diagnostic methods, based on DNA amplification known as PCR (Polymerase Chain Reaction) are promising tools in fast and specific detection and identification of biological agent(s). PCR is a technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA (9).

The R.A.P.I.D.TM-PCR (Ruggedized Advanced Pathogen Identification Device) is a 32 sample capacity, automated instrument integrating Idaho Technology's LightCycler® technology into a portable, impact resistant package. This allows field identification of pathogens quickly. The R.A.P.I.D. system is capable of automatically analyzing samples for the presence of any nucleic acid sequence, collect the data, interpret the test data and report the results. Automatic pathogen detection can be done in either "Batch" or "Screen" modes. Batch mode allows to test multiple samples for a single pathogen (two positive and two negative controls, and 28 unknown samples for given organisms). Screen mode allows testing for multiply organisms in a single run (two positive and two negative controls, and four unknown organisms, per test).

Monitoring the fluorescence from the double-stranded DNA dye (SYBR® Green) followed by differentiation of products by melting curves or from TaqMan® probes (6-FAM-oligo-TAMRA), allows inexpensive quantification of low initial template copy number. The fluorescent dye binds to the minor groove of the DNA double helix. In solution, the unbound dye exhibits very little fluorescence. The dye is very stable, only 6% of activity is lost during 30 amplification cycles. At the beginning of amplification, the reaction mixture contains the denatured DNA, the primers and the dye. The unbound dye molecules weakly fluorescence signal which is subtracted during computer analysis. After annealing of the primers, a few dye molecules can bind to the double strand. DNA binding results in a dramatic increase of the dye molecules to emit light upon excitation. During elongation, more and more dye molecules bind to the newly synthesized DNA.

Cycle sequencing reactions done in the thermocycling module of the R.A.P.I.D. system are faster, cleaner and more readable than parallel reactions done in a conventional heat block cycler. The use of air as the cycling medium ensures temperature uniformity and rapid heat exchange with the sample loaded in thin micro-capillary tubes, which are ideally suited to temperature cycling, because of their extremely high surface area to volume ratio. A conventional PCR protocol takes up-to 3 hours to do 30 three-temperature cycles. Thermal cyclers generally use micro centrifuge tubes or modified microtiter plates. These plastic containers insulate the sample. The temperature of a 100 μ l sample within a microcentrifuge tube lags 20-40 sec. behind the block temperature. This, combined with sluggish temperature ramp rates of heating blocks, explains why they are so slow.

The R.A.P.I.D. system can complete a 40-cycle reaction in less than 20 minutes (6 to 30min.). This makes R.A.P.I.D. system the fastest thermal cycler in the world. Rapid cool-down (up to 20 $^{\circ}$ C/sec) favors the kinetics of primer annealing over the thermodynamic advantage of product re-annealing. This in turn yields a higher final concentration of product. The most important benefit of rapid cycling is cleaner product. Rapid cycling leaves less time for primer extension at non-specific annealing. The amount of nonspecific product is directly related to the time spent at low temperatures.

There is another major factor - temperature holds times. A common protocol for temperature cycling is 1 min at 94 $^{\circ}$ C, 2 min at 55 $^{\circ}$ C and 3 min at 72 $^{\circ}$ C. Even if instantaneous temperature transitions were possible, one cycle would take 6 minutes. The actual enzymatic extension occurs in about 10 seconds for a 500 bp fragment. This leaves 97% of the total time wasted, not even counting ramp time. These machines are waiting for steady-state conditions to bring all the samples to roughly the same temperature. Unfortunately, because of the geometry of the heating elements and metal blocks, even the steady-state condition may not produce uniform temperatures across all samples. The R.A.P.I.D. uses micro-capillary tubes or thin walled microcentrifuge tubes, which are ideally suited to temperature cycling since they have very thin walls and extremely high surface area to volume ratio. Capillary tubes cut sample temperature lag to less than a second. The use of air as the cycling medium results in high temperature ramp rates. Tornado-like conditions inside the cylindrical reaction chamber ensure both temperature uniformity and rapid heat exchange with the sample. This allows the R.A.P.I.D. to spend less than one second at the high and low temperatures and still be certain that each sample has reached the target temperature. A fraction of a second adequate for the reaction.

The United States Air Force has developed over 50 assays for infectious agents on the R.A.P.I.D. system. Assays for infectious agents typically consist of two temperatures cycling for 40 cycles. Protocols for isolation of bacteria and viral DNA (or RNA) have been developed for clinical specimens, air samples and water samples. Protocols for food samples are being developed now. Assays in use for : *Bacillus anthracis*, *Yersinia pestis*, *Clostridium botulinum*, *Staphylococcus aureus*, *Francisella tularensis*, *Salmonella*, *Shigella*, *Vibrio cholerae*, *E. coli*, *Campylobacter*, *VEE*, *West Nile*, *Yellow Fever*, *Brucella spp.*, and many others.

CONCLUSIONS

- The R.A.P.I.D. system, as a fully automated, molecular diagnostic method, based on nucleic acid amplification and detection, is a promising tool in rapid identification of biological agents, which is essential for rapid response to BW attack.
- The continuing effort and advances in testing design make the R.A.P.I.D. system an asset for Army Commanders and Medical facilities alike.

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FIGURES AND TABLES

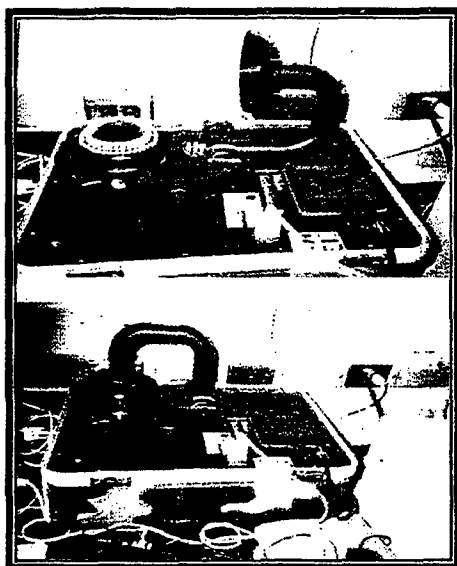


Fig.1. The R.A.P.I.D. system

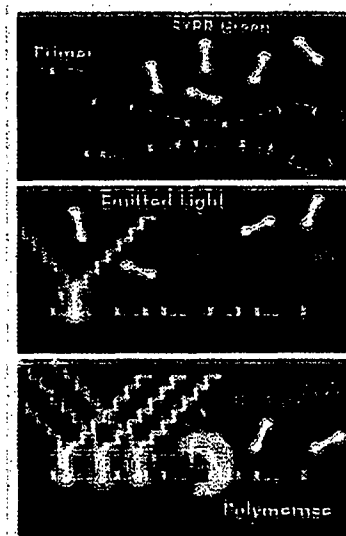


Fig. 2. Fluorescence